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Biochimica et Biophysica Acta 1502 (2000) 415–425

BIOCHIMICA ET BIOPHYSICA ACTA

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Mucopolysaccharidosis type IIIB: characterisation and expression of wild-type and mutant recombinant α-*N*-acetylglucosaminidase and relationship with Sanfilippo phenotype in an attenuated patient

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Received 22 May 2000; received in revised form 20 July 2000; accepted 20 July 2000

Abstract

Mucopolysaccharidosis type IIIB (MPS-IIB) is a lysosomal storage disorder characterised by the defective degradation of heparan sulfate due to a deficiency of α-*N*-acetylglucosaminidase (NAG). The clinical severity of MPS-IIIB ranges from an attenuated to severely affected Sanfilippo phenotype. This paper describes the expression and characterisation of wild-type recombinant NAG and the molecular characterisation of a previously identified R297X/F48L compound heterozygous MPS-IIIB patient with attenuated Sanfilippo syndrome. We have previously shown R297X to be the most common mutation in a cohort of Dutch and Australian patients, occurring at a frequency of approximately 12.5%. To date F48L has only been described in the proband. To determine the contribution of each mutation to the overall clinical phenotype of the patient, both mutant alleles were engineered into the wild-type NAG cDNA and expressed in Chinese hamster ovary cells. The wild-type NAG and F48L mutant alleles were also retrovirally expressed in MPS-IIIB skin fibroblasts. Residual NAG activity and the stability and maturation of immunoprecipitated NAG were determined for wild-type NAG and mutant NAG. The combined biochemical phenotypes of the two NAG mutant alleles demonstrated a good correspondence with the observed attenuated Sanfilippo phenotype of the patient.

Keywords: Lysosome; α-*N*-acetylglucosaminidase; Mucopolysaccharidosis; Sanfilippo

1. Introduction

The mucopolysaccharidoses are a group of 11 inherited lysosomal storage diseases (LSD) which result from deficiencies of specific lysosomal enzymes that are required for the lysosomal degradation of glycosaminoglycans (GAG). The mannose-6-phosphate (M6P) moieties on lysosomal enzymes serve as high-affinity ligands for binding to M6P receptors in the *trans*-Golgi network (TGN). Binding with

Abbreviations: MPS-IIIB, mucopolysaccharidosis type IIIB; NAG, α-*N*-acetylglucosaminidase; TGN, *trans*-Golgi network; WT-NAG, wild-type NAG; GAG, glycosaminoglycan; FCS, foetal calf serum; M6P, mannose-6-phosphate (M6P); CHO-KI, Chinese hamster ovary cells; DMEM, Dulbecco's modified Eagle's medium; F12, Ham's F12 medium

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0925-4439/00/$ – see front matter
PII: S0925-4439(00)00066-1

M6P receptors permits lysosomal enzymes to be segregated from proteins destined for secretion or transport to other intracellular compartments. The ligand-receptor complex is then transported from the TGN via a clathrin-coated vesicle to an acidified endosomal compartment where the M6P receptor and lysosomal enzyme dissociate. Further processing of the enzyme occurs in the lysosome. The M6P receptor is either recycled from the endosome back to the Golgi for further targeting of endogenous de novo synthesised lysosomal enzymes or transported to the plasma membrane where it functions to internalise exogenous lysosomal enzymes [1].

Mucopolysaccharidosis type III (MPS-III), also known as Sanfilippo syndrome, results from the absence of one of four lysosomal enzymes that are sequentially involved in the degradation of heparan sulfate: heparan *N*-sulfatase (MPS-IIIA), α-*N*-acetylglucosaminidase (NAG, MPS-IIIB), acetylCoA: α-glucosaminide acetyl-transferase (MPS-IIIC) or *N*-acetylglucosamine 6-sulfatase (MPS-IIID) [2]. An absence of any one of these enzymes leads to the lysosomal accumulation and urinary excretion of heparan sulfate and the clinical onset of Sanfilippo syndrome. In Australia the combined prevalence of MPS III is 1:66 000 births [3].

The clinical presentation of MPS-III is characterised by severe central nervous system degeneration resulting in progressive mental retardation and is often combined with hyperactivity and aggressive behaviour. The Sanfilippo phenotype of MPS-III varies, ranging from severe to attenuated. Unlike most of the other MPS, the somatic features of MPS-III, which involve skeletal pathology, hepatosplenomegaly and joint stiffness, are relatively mild and observed mainly in older patients. Often the lack of somatic involvement leads to difficulty in diagnosing patients with MPS-III [2].

The cDNA sequence for NAG has been cloned and characterised [4,5] with the gene localised to 17q21.1. Molecular characterisation of MPS-IIIB patients has resulted in the identification of several mutations in the NAG gene [5–11]. The high degree of molecular heterogeneity reflects the wide clinical variability observed with MPS-IIIB. Certain mutations show a higher prevalence. For example, the stop mutation R297X accounted for 12.5% of mutant alleles in a recent molecular study of 40 MPS IIIB patients (10 out of 80 alleles) [9]. Zhao et al. [11] reported a severely affected homozygous R297X/R297X Sanfilippo patient [11]. At 13 years of age the patient was severely demented, despite bone marrow transplantation. A severely affected homozygous R297X/R297X Sanfilippo patient, diagnosed at 6 years of age, has also been reported [9]. These observations suggest that R297X is associated with a severe Sanfilippo phenotype when inherited in a homozygous fashion [9,11]. The presence of an attenuated Sanfilippo phenotype in a F48L/R297X compound heterozygous patient suggests that the F48L allele is associated with an attenuated phenotype [9]. In this study the relationship between genotype, cell biochemistry and clinical phenotype in a F48L/R297X compound heterozygous Sanfilippo patient was evaluated by individually characterising the two mutations, F48L and R297X. As an initial step towards developing gene therapy for MPS-IIIB, we also retrovirally transduced MPS-IIIB skin fibroblasts with the NAG cDNA and demonstrated correction of the enzymatic and biochemical (GAG storage) defect.

2. Materials and methods

2.1. Case report: patient S.B.

Recently the genotype of the proband was determined to be compound heterozygous for R297X and F48L [9].

A 31-year-old male, born in 1968, presented at 18 months of age with failure to thrive, developmental delay, hepatomegaly and diarrhoea. He had three normal siblings. At age 3 years he had coarse hair, a protuberant abdomen, soft hepatomegaly and normal facies. His weight was less than the third percentile. A provisional diagnosis of glycogen storage disease was made.

Liver function testing and a skeletal survey were both normal. Liver biopsy was consistent with a lysosomal storage disorder likely to be MPS-III. A bone marrow biopsy and leucocytes showed lysosomal inclusions. A urinary screen for mucopolysaccharides (Royal Children's Hospital, Melbourne) was normal. A course of plasma infusions, then currently in vogue, was undertaken for 4 months. While this acutely shrank the liver and spleen, it was overall

without clinical effect. He remained intellectually delayed while displaying hyperactive aggressive behaviour but no regression.

As his course was atypical for MPS, repeat liver, skin and bone marrow biopsies were performed at age 8 years. At this stage there was persisting hepatomegaly, coarse hair and developmental delay. The liver biopsy was again consistent with a diagnosis of MPS-III with some progression to liver cirrhosis. The bone marrow inclusions persisted but a urinary screen for mucopolysaccharides remained negative. Skeletal survey showed minor vertebral changes. Aged 8, IQ testing put him in the 4- to 5-year range with a wide scatter. At this time he was living in a residential setting for the intellectually disabled. He attended a special school and was able to acquire some skills. Later he moved to a smaller institutional setting. Here he had a two-wheeler bike and used to ride freely around the neighbourhood to undertake a message or shopping. Whilst obviously intellectually delayed, he had a good memory for names and events and had become quite sociable.

At 12 years of age (1980) samples were sent to the Women's and Children's Hospital in North Adelaide, South Australia for high-resolution electrophoresis of urine [12] which showed a significant elevation of heparan sulfate, consistent with Sanfilippo syndrome. In contrast, his total urinary glycosaminoglycan content (19 mg/mmol creatinine) was just above the upper limit of normal for his age group (3–17 mg/mmol creatinine). These results emphasise the high incidence of false negative results in the urinary screening test for heparan sulfate by some methods leading to difficulty in diagnosing patients with MPS-III.

MPS-IIIB was diagnosed on the basis of the deficiency of NAG. Over the ensuing 8 years the patient did not show the rapid developmental deterioration associated with severe Sanfilippo syndrome. His liver was no longer enlarged and his joints were normal.

The third decade of life was characterised by a phase of intellectual deterioration and aggressive behaviour. He had an early loss of coordination aged 21 years and outbursts of rage, which improved after treatment with Thioridazine. Aged 22 years his memory deteriorated as did his self-care skills. He could no longer ride his bike and slept a lot regardless of Thioridazine dosage.

An echocardiogram performed aged 23 years was normal. Aged 23 years he underwent an inguinal hernia repair without complication. Nine months later he began to lose weight and develop paranoid delusions. He was noted to be prematurely ageing and engaging in meaningless babble. From the age of 24 years he seemed to lose his sense of direction and self care skills further deteriorated. This skill loss, along with memory loss became more profound from the age of 27 years. Presently aged 31, he has had a progressive loss of physical skills, co-ordination and muscle wasting manifested by frequent falls and inability to climb stairs. He is increasingly somnolent alternating with occasional erratic behaviour. He has poor short-term memory and no longer has self-care skills. He remains in residential care and still enjoys a reasonable quality of life.

2.2. Mutagenesis

The missense mutations F48L and R297X [9] were introduced into the WT NAG cDNA construct, pBluel33+4 [4] by using a QuickChange site-directed mutagenesis kit (Stratagene). The oligonucleotides used for each mutagenesis reaction are summarised in Table 1. Briefly, each polymerase chain reaction (PCR) (50 μl volume) contained 50 ng of pBluel33+4 template, 125 ng of each oligonucleotide primer (Table 1), 1×reaction buffer, 1 μl of dNTP mix, 1×Q buffer (Qiagen) and 2.5 units of Pfu Turbo DNA polymerase. Each PCR was then denatured at 95°C for 30 s and then subjected to 12 cycles of the following conditions: 95°C for 30 s, 55°C for 1 min and 68°C for 12 min. Ten units of *Dpn*I restriction enzyme was then added to each reaction and incubated at 37°C for 1 h. Five μl of each *Dpn*I digested PCR was then transformed into Epicurian Coli XLI-Blue Supercompetent cells as described by the supplier (Stratagene). Clones containing the mutated sequence were identified by allele-specific oligonucleotide hybridisation (Table 1) and subsequently sequenced to ensure that no other changes other than the desired mutation had been introduced into the NAG cDNA sequence. pBluel33+4*F48L* and pBluel33+4*R297X* were digested with *Bst*EII and *Bam*HI. The inserts containing the mutagenised NAG sequences were then sub-cloned into the WT NAG expression construct pcDNA*NAG* [4] digested

Table 1
Sequence of oligonucleotide primers that were used for the quick change mutagenesis of WT-NAG cDNA sequence

Primer pair	Sequence	Sense or antisense	Position
F48L-1	CCGCGGCCGACCTCTCCGTGTCG	+	232–254
F48L-2	CGACACGGAGAGGTCGGCCGCGG	−	254–232
R297X-1	CGGAGCCTCTTCCTGTGAGAGCTGATCAAAG	+	975–1006
R297X-2	CTTTGATCAGCTCTCACAGGAAGAGGCTCCG	−	1006–975

The positions of primers in the NAG sequence are according to Fig. 3 of Weber et al. [4].

with the same restriction endonucleases. Clones containing the mutagenised NAG sequence were identified by allele specific oligonucleotide hybridisation and designated pcDNA*F48L* and pcDNA*R297X*.

2.3. Cell culture

Unless otherwise stated, all cell culture reagents were purchased from Gibco-BRL. Chinese hamster ovary cells (CHO-KI) were grown in Ham's F12 (F12) supplemented with 10% (v/v) bovine foetal calf serum (FCS, CSL Ltd), and antibiotics (penicillin, 100 U/ml and streptomycin sulfate, 100 μg/ml). Skin fibroblasts, Bosc 23 cells [13] and PA317 cells [14] were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with FCS (10% v/v) and antibiotics.

2.4. Electroporation of CHO-KI cells

CHO-KI cells were electroporated with 30 μg of pcDNA*F48L*, pcDNA*R297X*, and pcDNA*NAG* as previously described [15]. Electroporated cells were selected with 0.75 mg/ml geneticin (G418) for 2 weeks resulting in the appearance of approximately 300 G418-resistant colonies per electroporation. G418-resistant mass-cultures of electroporated cells were used for the determination of NAG activity and immunoprecipitation (see below).

2.5. Retroviral vector construction

The WT NAG cDNA or mutagenised NAG sequence was excised from pcDNA*NAG* and pcDNA*F48L* with *Hin*dIII and *Eco*RV and subcloned into pLNCX [16] digested with *Hin*dIII and *Stu*I. The presence of the NAG cDNA in each construct was confirmed by restriction enzyme analysis. Constructs containing the cDNA in the correct orientation were isolated and designated pLNC*NAG* and pLNC*F48L*.

2.6. Generation of amphotropic LNCNAG and LNCF48L virus

The retroviral constructs pLNC*NAG* and pLNC*F48L* (25 μg) were transfected into the ecotropic virus packaging cell line Bosc-23 [13] using Lipofectamine transfection reagent (Gibco-BRL). Medium containing transiently expressed ecotropic retrovirus was harvested 48–96 h after transfection, filtered (0.2-μm filter) and used to infect PA317 cells [14] in the presence of 4 μg/ml polybrene. PA317 cells were exposed to four 12-h cycles of freshly harvested ecotropic virus infection, then subcultured 1:3 and grown in the presence of 1 mg/ml G418 for 2 weeks.

2.7. Retrovirus transduction of MPS-IIIB skin fibroblasts

Sixteen-hour-conditioned medium was harvested from G418-resistant mass-cultures of PA317/LNC*F48L* and PA317/LNC*NAG*, filtered (0.2-μm filter) and used to infect MPS-IIIB (R297X/R297X homozygous) skin fibroblasts (SF 4657, 70% confluent in 75-cm^2 flasks) in the presence of 4 μg/ml polybrene. MPS-IIIB fibroblasts were exposed to five cycles of freshly harvested amphotropic virus infection. Each virus exposure was approximately 16–24 h. After the final virus exposure the transduced cells were fed with growth medium until they reached confluence. Transduced fibroblasts were then subcultured 1:3 into three 75-cm^2 flasks and grown in the presence of 0.3 mg/ml G418 for 3 weeks.

2.8. Determination of NAG activity

Skin fibroblast and CHO-KI cultures were grown

to confluence in 75-cm^2 flasks. At 3 days post-confluence the cells were harvested with trypsin-versene (CSL Ltd), washed with PBS, resuspended in 150 μl 0.5 M NaCl/0.02 M Tris (pH 7) and subjected to seven cycles of freeze–thaw in a slurry of dry ice and ethanol. Lysates were clarified by microcentrifugation at 13 000 rpm for 3 min. Clarified fibroblast lysates and CHO-KI lysates were assayed for NAG activity using the fluorogenic substrate 4-methylumbelliferyl-2-acetomido-2-deoxy-α-D-glucopyranoside (Calbiochem) [17] and normalised for protein concentration as determined by the BioRad protein assay (BioRad).

2.9. Analysis of GAG storage

Normal fibroblasts (SF 4858), MPS-IIIB fibroblasts (R297X/R297X homozygous; SF 4657), LNC*F48L* and LNC*G737R* transduced MPS-IIIB fibroblasts were grown to confluence in 25-cm^2 flasks and then metabolically labelled for 24 h with 10 μCi/ml $Na_2^{35}SO_4$ (570 mCi/mmol; DuPont NEN) in antibiotic-free Ham's F12 supplemented with 10% (v/v) FCS. Labelled cells were then harvested with trypsin-versene (CSL Ltd) and transferred to new 25-cm^2 flasks containing DMEM supplemented with 10% FCS and antibiotics (penicillin, 100 U/ml and streptomycin sulfate, 100 μg/ml) and grown for a further 5 days. Some flasks of labelled MPS-IIIB fibroblasts were exposed to 24-h-conditioned medium collected from LNC*NAG* transduced MPS-IIIB fibroblasts in the presence or absence of 5 mM M6P (Sigma) during the 5-day chase period. Cell lysates were prepared and assayed for total cell protein, ^{35}S radioactivity, β-hexosaminidase and NAG activity.

2.10. Immunoprecipitation of NAG

CHO-KI cells and G418-resistant mass-cultures of CHO*R297X*, CHO*F48L* and CHO*NAG* were grown to confluence in 75-cm^2 flasks and then metabolically labelled for 30 min with 100 μCi/ml EXPRE^{35}S^{35}S [^{35}S] protein labelling mix (1175 Ci/mmol, DuPont, NEN) in cysteine-methionine-free DMEM (ICN Biomedicals) supplemented with L-glutamine and FCS. Labelled cells were rinsed with medium and then chased with growth medium (Ham's F12 supplemented with FCS and antibiotics) for 4, 11 or 16 h. At each chase time one 75-cm^2 flask of labelled cells were harvested by treatment with trypsin-versene, recovered by centrifugation, resuspended in 10 ml PBS and then centrifuged again. The cell pellet was resuspended in 10 ml of 1×solubilisation buffer (PBS containing 1% w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulfate (SDS), 0.5% Nonidet P-40) and incubated for 24 h at 4°C. Prior to immunoprecipitation with NAG polyclonal antisera, growth medium and solubilised cell lysates were pre-cleared as follows. Seventy μl of polyclonal CHO-KI antiserum (prepared by CSL Ltd) was added to each medium or solubilised lysate sample and incubated at 4°C overnight. Equilibrated pansorbin cells (140 μl, Calbiochem) were added to each sample, incubated at 4°C for a further 6 h and then sedimented by centrifugation. The pre-cleared supernatant was then immunoabsorbed using 40 μl of unpurified rabbit polyclonal antisera raised against recombinant NAG (Weber et al., submitted). After an overnight incubation at 4°C, 70 μl of equilibrated pansorbin cells were added to each sample and incubated at 4°C for 6 h. NAG–IgG–pansorbin complexes were

Table 2
Expression of F48L-NAG, R297X-NAG and WT-NAG in CHO-KI cells

	Intracellular NAG (nmol/h per mg)	Secreted NAG (nmol/h per ml)	Intracellular β-hex (nmol/min per mg)
CHO-KI	7.81 ± 0.37	4.32 ± 0.24	19.31 ± 0.95
CHO*F48L*	8.23 ± 0.44	4.53 ± 0.18	21.76 ± 0.36
CHO*R297X*	7.85 ± 0.17	4.67 ± 0.18	16.49 ± 0.16
CHO*NAG*	159.63 ± 4.76	13.20 ± 0.27	19.67 ± 0.35

CHO-KI cells were electroporated with the mutagenised or WT-NAG cDNA. Twenty-four-hour-conditioned medium and cell lysates prepared from confluent G418-resistant mass cultures were assayed for NAG activity or β-hexosaminidase (β-hex) activity using fluorogenic substrates. Results are expressed as the mean ± S.D. (n = 3).

pelleted by centrifugation, washed by microcentrifugation/resuspension with two 1-ml volumes of solubilisation buffer, once with 1 ml of water and then resuspended in 50 μl sample buffer. Samples were boiled for 5 min, centrifuged at 12 000 × *g* for 1 min to pellet Pansorbin cells, and the supernatant analysed via SDS–polyacrylamide gel electrophoresis (PAGE) and autoradiography.

3. Results

3.1. Expression of F48L-NAG, R297X-NAG and WT-NAG in CHO-KI cells

G418-resistant mass-cultures of CHO-KI cells over-expressing F48L-NAG, R297X-NAG and WT-NAG were assayed for NAG activity using the fluorogenic substrate as described in Section 2. Control CHO-KI cells contained a significant amount of endogenous NAG activity (7.81 nmol/h per mg total cell protein, Table 2). CHO-KI cells electroporated with pcDNA*F48L* (CHO*F48L*) and pcDNA*R297X* (CHO*R297X*) contained NAG activities similar to control CHO-KI cells (8.23 nmol/h per mg and 7.85 nmol/h per mg, respectively). Medium harvested from control CHO-KI, CHO*F48L* and CHO*R297X* cells contained comparable NAG activities as well (Table 2). In contrast, in G418-resistant mass-cultures of CHO-KI cells electroporated with pcDNA-NAG (CHO*NAG*), significant amounts of NAG activity toward the fluorogenic substrate were measured (159 nmol/h per mg, Table 2). Medium harvested from CHONAG cells contained 13.2 nmol/h per ml of NAG activity (Table 2). The activity of the lysosomal enzyme β-hexosaminidase was not affected by the expression of F48L-NAG, R297X-NAG and WT-NAG in CHO-KI cells (Table 2).

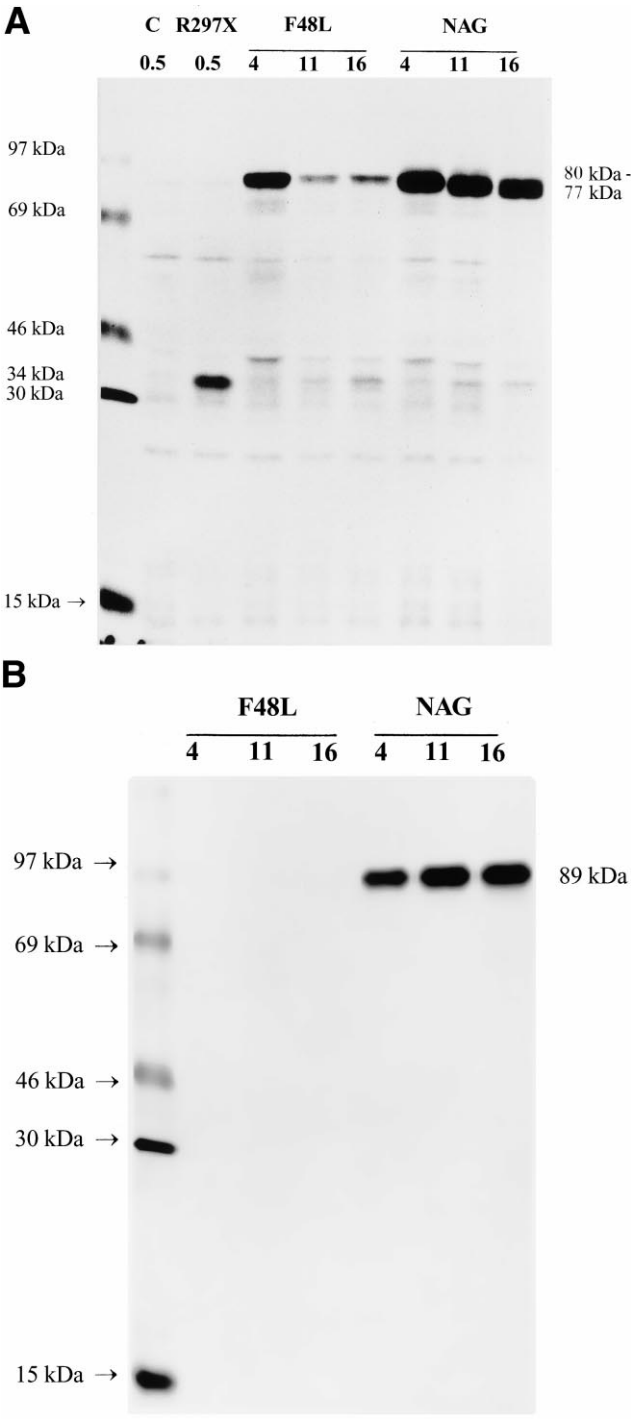

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Fig. 1. Stability of intracellular (A) and secreted (B) R297X-NAG, F48L-NAG and WT-NAG in CHO-KI cells. Control CHO-KI cells (C), CHO*R297X* cells (R297X), CHO*F48L* (F48L) and CHO*NAG* (NAG) cells were grown to confluence in 75-cm^2 tissue-culture flasks and metabolically labelled with EXPRE^{35}S^{35}S [^{35}S] protein labelling mix for 30 min. The labelling medium was then removed and cells were chased in growth medium for 0.5 h (CHO-KI and CHO*R297X* cells), or 4, 11 and 16 h (CHO*F48L* and CHO*NAG* cells). At each time point labelled cells (A) and growth medium (B) were harvested, immunoprecipitated and analysed via SDS–PAGE and autoradiography. The molecular masses are indicated with arrows and were calculated by comparison with C-14 methylated standards run alongside the gel.

Table 3
Analysis of GAG storage in retrovirus transduced MPS-IIIB fibroblasts

	Intracellular NAG (nmol/h per mg cell protein)	Secreted NAG (nmol/h per ml)	Intracellular β-hex (nmol/min per mg)	^{35}S Radioactivity (cpm/mg cell protein)
Normal fibroblasts	8.815 ± 0.175	0.5 ± 0.3	46.9 ± 2.2	6,975 ± 991
MPS-IIIB fibroblasts	n.d.	n.d.	68.1 ± 0.5	84,351 ± 510
MPS-IIIB/LNC*F48L*	1.86 ± 0.07	n.d.	48.3 ± 0.8	55,711 ± 1576
MPS-IIIB/LNC*NAG*	49.6 ± 0.21	46.6 ± 0.4	49.7 ± 0.5	12,461 ± 1230

MPS-IIIB skin fibroblasts were retrovirally transduced with F48L-NAG (MPS-IIIB/LNC*F48L*) or WT-NAG (MPS-IIIB/LNC*NAG*) as described in Section 2. Control normal fibroblasts, control MPS-IIIB fibroblasts or gene-transduced fibroblasts were metabolically labelled for 24 h with $Na_2^{35}SO_4$ and then passaged for a further 3 days. Cell lysates were prepared and assayed for total protein, ^{35}S radioactivity, β-hexosaminidase (β-hex) activity and NAG activity. Twenty-four-hour-conditioned medium was also collected from each cell line and assayed for NAG activity. Results are expressed as the mean ± S.D. ($n = 3$). n.d., not detected.

3.2. Immunoprecipitation of R297X-NAG, F48L-NAG and WT-NAG

Control CHO-KI cells and G418-resistant mass-cultures of CHO*R297X* were metabolically labelled with EXPRE^{35}S^{35}S [^{35}S] protein labelling mix for 30 min and then chased for 30 min or 4 h. At the 30-min chase time point ^{35}S-labelled R297X-NAG immunoprecipitated from CHO*R297X* cell lysates was detected as a truncated 34 kDa polypeptide (Fig. 1A) which was no longer detectable at the 4-h chase time point (result not shown). No ^{35}S-labelled R297X NAG was detectable in the chase medium (result not shown).

G418-resistant mass-cultures of CHO*F48L* and CHO*NAG* were metabolically pulse-labelled with EXPRE^{35}S^{35}S [^{35}S] protein labelling mix for 30 min and then chased for varying lengths of time (4, 11 or 16 h). At the 4-h chase time point, ^{35}S-labelled WT-NAG immunoprecipitated from CHO*NAG* cell lysates migrated under reducing conditions as a precursor 80 kDa polypeptide. At the 11- and 16-h chase time points intracellular WT-NAG was successively reduced to 78.5 and 77 kDa, respectively (Fig. 1A). At the 4-, 11- and 16-h chase time points, the secreted form of ^{35}S-labelled WT-NAG was detected as a precursor 89 kDa polypeptide (Fig. 1B). In contrast, ^{35}S-labelled F48L-NAG immunoprecipitated from CHO*F48L* cell lysates was only detected as a 80 kDa precursor polypeptide (Fig. 1A). F48L-NAG did not appear to be cleaved to the intracellular mature 77 kDa form or be secreted into the medium (Fig. 1A,B).

3.3. Expression of F48L-NAG and WT-NAG in MPS-IIIB skin fibroblasts

MPS-IIIB fibroblasts were exposed to five cycles of

Table 4
Partial correction of GAG storage in MPS-IIIB fibroblasts with MPS-IIIB/LNC*NAG* conditioned medium

	NAG (nmol/h per mg)	β-Hexosaminidase (nmol/min per mg)	^{35}S Radioactivity (cpm/mg cell per protein)
Normal fibroblasts	25.12 ± 2.23	84.3 ± 14.7	3915 ± 182
MPS-IIIB fibroblasts	0.101 ± 0.025	94.0 ± 12.7	47988 ± 3328
MPS-IIIB fibroblasts+MPS-IIIB/LNC*NAG* conditioned medium	0.388 ± 0.048	80.7 ± 4.4	33305 ± 1518
MPS-IIIB fibroblasts+MPS-IIIB/LNC*NAG* conditioned medium+5 mM M6P	0.057 ± 0.012	94.5 ± 4.5	48874 ± 3861

Confluent triplicate cultures (25-cm^2 flasks) of normal fibroblasts (row 1) and MPS-IIIB fibroblasts (rows 2–4) were labelled for 24 h with $Na_2^{35}SO_4$ then passaged for a further 5 days. Some groups of labelled MPS-IIIB fibroblasts (rows 3 and 4) were also exposed to conditioned medium (46.6 nmol/h per ml) collected from LNC*NAG*-transduced MPS-IIIB fibroblasts in the absence (row 3) or presence (row 4) of 5 mM M6P during the 5-day chase period. Cell lysates were prepared and assayed for total protein, ^{35}S radioactivity, β-hexosaminidase and NAG activity. Results are expressed as the mean ± S.D. ($n = 3$).

LNC*F48L* or LNC*NAG* virus infection and then passaged for 3 weeks in the presence of 0.3 mg/ml G418. The storage of undegraded, sulfated GAGs in MPS-IIIB fibroblasts was analysed by measuring the intracellular retention of radioactivity in cells metabolically labelled with $Na_2^{35}SO_4$. In this experiment R297X/R297X homozygous skin fibroblasts obtained from a severely affected MPS-IIIB patient (patient 30 of Table 4, Weber et al. [9]) contained no detectable NAG activity and approximately 12 times more intracellular ^{35}S radioactivity than normal control fibroblasts (Table 3). We have made the assumption that the majority of accumulated intracellular ^{35}S radioactivity in MPS-IIIB fibroblasts corresponds to the stored GAG, heparan sulfate. Transduction of MPS-IIIB fibroblasts with LNC*NAG* virus resulted in the clearance of ^{35}S-labelled GAG storage to amounts approaching normal (Table 3). The amount of intracellular NAG activity in LNC*NAG*-transduced fibroblasts was elevated 5.6-fold when compared with normal fibroblasts (Table 3). In contrast, the amount of intracellular NAG activity in LNC*F48L* transduced MPS-IIIB fibroblasts was significantly reduced (1.86 nmol/h per mg) and corresponded to 3.75% of NAG activity in LNC*NAG*-transduced MPS-IIIB fibroblasts (Table 3). The residual amount of NAG activity in LNC*F48L*-transduced MPS-IIIB fibroblasts resulted in the partial clearance of ^{35}S-labelled GAG storage (55 711 cpm/mg, Table 3) when compared with ^{35}S-labelled GAG storage in control MPS-IIIB fibroblasts (84 351 cpm/mg, Table 3). The expression of the lysosomal enzyme β-hexosaminidase was not affected by the retroviral-mediated gene expression of F48L-NAG and WT-NAG.

Conditioned medium harvested from LNC*NAG*-transduced MPS-IIIB fibroblasts contained 46.6 nmol/h per ml of NAG activity (Table 3). To determine whether this secreted form of NAG was being correctly processed, two groups of MPS-IIIB fibroblasts were incubated with 24-h-conditioned medium harvested from LNC*NAG*-transduced fibroblasts (46.6 nmol/h per ml, Table 3). Incubation with conditioned medium from LNC*NAG*-transduced MPS-IIIB fibroblasts resulted in the partial clearance of ^{35}S-labelled GAG storage and the detection of only a small amount of endocytosed NAG activity in MPS-IIIB fibroblasts (Table 4). The presence of 5 mM M6P in the culture medium prevented the uptake of recombinant NAG by MPS-IIIB fibroblasts and clearance of ^{35}S-labelled GAG storage (Table 4).

3.4. Analysis of I-cell plasma

Plasma from I-cell patients contained elevated amounts of NAG and β-hexosaminidase when compared with normal control plasma samples. However, acid phosphatase, which is targeted to the lysosome independently of the mannose-6-phosphate receptor pathway, was not elevated in I-cell plasma samples when compared with normal control plasma samples (Table 5).

4. Discussion

4.1. Expression of R297X-NAG and F48L-NAG

In this study we investigated the relationship between genotype, cellular biochemistry and clinical phenotype in a F48L/R297X compound heterozygous MPS-IIIB patient with an attenuated Sanfilippo phenotype. Molecular characterisation of this compound heterozygous MPS-IIIB patient was complicated by the presence of two mutant NAG alleles, which together cause an attenuated Sanfilippo phenotype (see Section 2.1). To determine the effect of each mutation on NAG synthesis, sub-cellular distribution and activity, both mutant alleles were engi-

Table 5
Analysis of β-hexosaminidase, NAG and acid phosphatase activity in I-cell plasma

	β-Hexosaminidase (nmol/min per ml)	NAG (nmol/h per ml)	ACP (nmol/min per ml)
Control plasma ($n=3$)	5.83 ± 1.58	19.7 ± 6.3	21.67 ± 5.78
I-cell plasma ($n=3$)	96.1 ± 10.73	316.93 ± 32.36	21.33 ± 3.56

Plasma from normal controls (37, 38, 39) or I-cell patients (285, 256, 257) were assayed for β-hexosaminidase, NAG or acid phosphatase (ACP) using fluorogenic substrates. Results are expressed as the mean ± S.D. ($n=3$)

neered into the wild-type NAG cDNA and expressed. The R297X allele displayed no detectable NAG activity when expressed in CHO-KI cells and was observed only as a 34 kDa truncated polypeptide that was rapidly degraded. Skin fibroblasts obtained from a homozygous R297X/R297X patient contained very low levels of NAG activity (0.101 nmol/h per mg total protein, Table 4) and a 12-fold elevated level of ^{35}S-labelled GAG storage when compared with normal fibroblasts. The low levels of NAG activity detected in these fibroblasts may be due to bovine NAG activity derived from the foetal calf serum present in the culture medium or alternatively may be due to a small amount of translational read-through of the R297X mutant allele. The highly elevated levels of ^{35}S-labelled GAG storage in homozygous R297X/R297X skin fibroblasts are consistent with the clinically severe phenotype associated with the R297X allele [9,11].

When over-expressed in CHO-KI cells and analysed via immunoprecipitation, F48L-NAG was detected as a 80 kDa precursor polypeptide that was not cleaved to the mature 77 kDa form or secreted into the medium. Furthermore, the 80 kDa F48L-NAG precursor polypeptide was partially degraded over the 16-h chase period (Fig. 1A). These results suggest that the F48L missense mutation affects the processing and stability of NAG. The F48L precursor polypeptide may correspond to newly synthesised protein that is selectively retained in the endoplasmic reticulum due to incorrect folding and is subsequently degraded. Alternatively, some F48L-NAG may be synthesised as an unstable precursor polypeptide that is transported to the lysosome and rapidly degraded in the hydrolytic environment. When expressed via retroviral-mediated gene transfer in homozygous R297X/R297X MPS-IIIB skin fibroblasts, intracellular F48L-NAG activity corresponded to 3.75% of NAG activity levels in WT-NAG-transduced MPS-IIIB fibroblasts. This residual level of NAG activity was sufficient to metabolise 34% of intracellular ^{35}S-labelled GAG storage suggesting that some F48L-NAG is being correctly sorted to the lysosomal compartment. The residual level of F48L-NAG activity in the lysosomes of F48L-NAG-transduced MPS-IIIB fibroblasts and subsequent partial turnover of GAG is consistent with the attenuated clinical phenotype associated with the F48L allele in patient SB (see Section 2.1).

4.2. Expression of WT-NAG

The WT-NAG cDNA was over-expressed in CHO-KI cells and immunoprecipitated with a rabbit polyclonal antibody to recombinant NAG. To characterise WT-NAG in terms of its maturation, newly synthesised radiolabelled NAG was immunoprecipitated after varying lengths of chase time. Intracellular NAG was detected as a precursor polypeptide of 80 kDa which was successively cleaved to a 78.5 kDa intermediate polypeptide and a mature 77 kDa polypeptide over a 16-h chase time (Fig. 1A). This is in agreement with previous studies in which native NAG has been purified to homogeneity from a number of different tissues ([4,11,18]). The delayed processing of newly synthesised NAG in CHO-KI cells may be expression system-specific rather than NAG specific as we have also observed slow processing of other lysosomal enzymes in CHO-KI cells [19].

As an initial step toward gene therapy in animal models for MPS-IIIB, a retroviral construct that contained WT-NAG cDNA sequence was made (pLNC*NAG*) and used to transduce cultured MPS-IIIB skin fibroblasts. The pLNC*NAG* construct was designed to express NAG from the cytomegalovirus immediate–early (CMV) promoter and the neomycin-resistance gene from the viral LTR promoter. Amphotropic LNC*NAG* virus was generated and used to transduce MPS-IIIB skin fibroblasts. G418-resistant mass cultures of LNC*NAG*-transduced MPS-IIIB fibroblasts displayed correction of the enzymatic and storage phenotype associated with MPS-IIIB (Table 3). These results suggest that LNC*NAG* gene-corrected MPS-IIIB fibroblasts express functional NAG that is correctly processed and targeted to the lysosome. Although there was a fivefold elevated level of NAG activity in LNC*NAG*-transduced MPS-IIIB fibroblasts when compared with normal control fibroblasts, ^{35}S-labelled GAG storage was not completely normalised. This could be due to slightly different growth rates of the normal control fibroblasts and LNC*NAG*-transduced MPS-IIIB fibroblasts, different rates of incorporation of the ^{35}S-label into GAGs or different amounts of GAG production by the two groups of cells.

LNC*NAG*-transduced MPS-IIIB fibroblasts secreted detectable levels of NAG activity into the culture medium (46.6 nmol/h per ml, Table 3). This conditioned medium was used in a cross-correction experiment to determine whether the secreted form of WT-NAG was correctly mannose-6-phosphorylated. Incubation of MPS-IIIB skin fibroblasts for an extended chase period (5 days) with conditioned medium from LNC*NAG*-transduced MPS-IIIB fibroblasts resulted in only a small amount of endocytosed intracellular NAG activity which was sufficient to partially reduce ^{35}S-labelled GAG storage (Table 4). No clearance of ^{35}S-labelled GAG storage was observed with shorter chase periods (3 days, results not shown). In the presence of 5 mM M6P no reduction in ^{35}S-labelled GAG storage was observed. These results suggest that the majority of recombinant WT-NAG secreted from LNC*NAG*-transduced MPS-IIIB fibroblasts is not mannose-6-phosphorylated. Only a small proportion (0.36%) of WT-NAG secreted from LNC*NAG*-transduced MPS-IIIB fibroblasts is correctly phosphorylated. This small amount of secreted, phosphorylated WT-NAG can be endocytosed by a second population of MPS-IIIB skin fibroblasts via the M6P receptor pathway and be correctly targeted to the lysosome (Table 4).

We have not observed weak phosphorylation of other secreted recombinant lysosomal enzymes expressed in CHO-KI cells or in patient cell lines ([15,19–21]). When directly compared with another recombinant lysosomal enzyme over-expressed in CHO-KI cells, *N*-acetylgalactosamine-4-sulfatase (CHO4S), the M6P receptor-mediated uptake of CHONAG into cultured skin fibroblasts was considerably reduced (Weber et al., unpublished results). Furthermore, in previous cross-correction experiments using *N*-acetylgalactosamine-4-sulfatase gene transduced MPS-VI skin myoblasts, 8.9% (0.664 pmol/min) of the total *N*-acetylgalactosamine-4-sulfatase secreted from the gene-corrected myoblasts (7.45 pmol/min) was endocytosed by a second population of MPS-VI myoblasts via the M6P receptor pathway [22]. In contrast, in this study only 0.36% (1.19 nmol/h) of the total NAG secreted from LNC*NAG*-transduced MPS-IIIB fibroblasts (329 nmol/h) was endocytosed by a second population of MPS-IIIB fibroblasts via the M6P receptor pathway (Table 4). Weak phosphorylation of secreted rNAGLU expressed in HeLa cells has also been observed (G. Yogalingam, unpublished results). These results suggest that weak phosphorylation of secreted WT-NAG is NAG-specific rather than expression system-specific. The causative factors which lead to poor mannose-6-phosphorylation of secreted WT-NAG were not investigated further in this study. We did not determine whether the poor phosphorylation of WT-NAG is due to only a small degree of the enzyme being post-translationally phosphorylated or due to the enzyme being phosphorylated normally but susceptible to very rapid dephosphorylation. These results suggest that the use of secreted recombinant WT-NAG in future enzyme and gene therapy protocols will be severely limited due to its small degree of mannose-6-phosphorylation.

The importance of targeting lysosomal enzymes via the M6P receptor pathway is demonstrated in patients with I-cell disease. Due to a deficiency of UDP-*N*-acetylgalactosamine phosphotransferase, I-cell patients are unable to transfer M6P moieties onto carbohydrate side chains of lysosomal enzymes. The presence of elevated NAG activity in I cell plasma samples (Table 5) indicates that normally the intracellular form of WT-NAG is correctly phosphorylated and targeted to the lysosome. The results obtained in this study have demonstrated that LNC*NAG*-transduced MPS-IIIB fibroblasts express intracellular WT-NAG that is correctly targeted to the lysosome and capable of turning over stored GAG. Although the secreted form of WT-NAG is poorly mannose-6-phosphorylated, these initial studies do highlight the potential for using the correctly mannose-6-phosphorylated, intracellular form of recombinant WT-NAG in future gene therapy treatments for MPS-IIIB patients.

Acknowledgements

We thank Drs Donald Anson and Thomas Berg for helpful discussion on the immunoprecipitation and site-directed mutagenesis methods used in this study. The authors are grateful to Ms Kaye Beckman for the purification of recombinant NAG used for the generation of rabbit polyclonal antisera and to Ms Xiao Hui Guo for sequencing the mutagenised

NAG constructs. Polyclonal CHO-KI antiserum was prepared by CSL Ltd. I-cell and control plasma samples were a generous gift from the National Referral Laboratory.

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